

**THE EFFECT OF MUSHROOM EXTRACTS ON CYTOSOLIC ENZYMES  
COMBINED WITH SELECTED DRUG: DOXORUBICIN**

**A MASTER'S THESIS**

**IN**

**CHEMICAL ENGINEERING AND APPLIED CHEMISTRY DEPARTMENT**

**ATILIM UNIVERSITY**

**BY**

**MOHAMED M. ALWERFALLY**

**OCTOBER 2014**

**THE EFFECT OF MUSHROOM EXTRACTS ON CYTOSOLIC  
ENZYMES COMBINED WITH SELECTED DRUG:  
DOXORUBICIN**

**A THESIS SUBMITTED TO  
THE GRADUATE SCHOOL OF NATURAL AND APPLIED  
SCIENCES**

**OF ATILIM UNIVERSITY**

**BY**

**MOHAMED M. ALWERFALLY**

**IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR**

**THE DEGREE OF**

**MASTER OF SCIENCE INAPPLIED CHEMISTRY**

**OF**

**CHEMICAL ENGINEERING AND APPLIED CHEMISTRY  
DEPARTMENT**

**OCTOBER 201**

Approval of the Graduate School of Natural and Applied Sciences, Atılım University.

---

(Prof. Dr. İbrahim K. Akman )

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

---

(Prof. Dr. Atilla Cihaner )

Head of Department

This is to certify that we have read the thesis “The Effect Of Mushroom Extracts on Cytosolic Enzymes Combined With Selected Drug: Doxorubicin” submitted by “Mohamed Alwerfally” and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

---

(Asst. Prof. Dr. S. Belgin İşgör)

Supervisor

Examining Committee Members

Prof. Dr. Özlem Yildirim (Chairman)

Prof. Dr. Atilla Cihaner

Assoc. Prof. Dr. İlgez Akara

Asst. Prof. Dr. Murat Kaya

I declare and guarantee that all data, knowledge and information in this document has been obtained, processed and presented in accordance with academic rules and ethical conduct. Based on these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Mohamed Alwerfally

Signature:

## **ABSTRACT**

### **THE EFFECT OF MUSHROOM EXTRACTS ON CYTOSOLIC ENZYMES COMBINED WITH SELECTED DRUG: DOXORUBICIN**

**Alwerfally, Mohamed M.A.**

**M.Sc., Department of Chemical Engineering and Applied Chemistry  
Applied Chemistry Master Program**

**Supervisor: Assist. Prof. Dr. S. Belgin İsgör  
October 2014, Page: 39**

Recently many research groups have proposed that mushrooms and their extracts while using as a food supplement during chemotherapy increased the drug effect and also lifespan of the patients. The most frequently studied family of the mushrooms to evaluate their effect against cancer is the Reishi mushrooms. Since it has been known that most of the antioxidant enzymes show resistance against cancer drugs in this study, the effect of mushroom extracts on the following antioxidant enzymes; glutathione-S-transferase, glutathione peroxidase and catalase with the presence and the absence of the cancer drug Doxorubicin were compared. The mushroom extracts were prepared by using two different solvents of methanol and acetone separately and used for further analysis. The results of the extracts solvents were also compared.

The results showed that the effect of mushroom extracts with Doxorubicin was greater than that of the mushroom extracts alone. The inhibition of Glutathione-S-transferase (GST) was 45% by mushroom extracted in acetone and Doxorubicin while it was 30% by pure mushroom extract. In Catalase (CAT) assay the inhibition was 45% by acetone mushroom extract containing Doxorubicin and it was 35% by acetone extracted mushroom. In Glutathione peroxidase (GPX) the inhibition was 15% by acetone extracted mushroom with Doxorubicin while it was 35% by only acetone extracted mushroom. In methanol extracted mushroom there is no significant results in the most of the assays.

## ÖZ

### **MANTAR ÖZÜTLERİNİN DOXORUBİCİN İLACI İLE KOMBİNASYONUNUN SİTOZOLİK ENZİMLER ÜZERİNE ETKİSİ**

**Alwerfally, Mohamed M.A**

**Yüksek Lisans: Kimya Mühendisliği ve Uygulamalı Kimya Bölümü**

**Uygulamalı Kimya Yüksek Lisans Programı**

**Danışman: Yrd. Doç. Dr. S. Belgin İşgör**

**Sayfa: 39**

Son zamanlarda pek çok araştırma grubu tarafından yapılan çalışmalarda belirli türlerde mantar ve bu mantarlara ait özlerin kemoterapi sırasında gıda takviyesi olarak kullanıldığında kemoterapinin etkisini artırdığı ve hastaların yaşam sürelerini uzattığı gösterilmiştir. Bu konuda en fazla çalışılan mantar türlerinden biri de Reishi mantarıdır. Antioksidan enzimlerin pekçoğunun kanser ilaçlarına karşı direnç gösterdiği bilinmektedir. Bu nedenle bu çalışmada mantar ekstraktlarının glutathione-S-transferaz, glutathione peroksidaz ve katalaz antioksidan enzimleri üzerine etkisine kanser ilacı olan Doxorubicin varlığında ve yokluğunda bakılmıştır. Çalışmada hazırlanan mantar ekstraktları iki ayrı çözücü kullanılarak hazırlanmış ve sonuçlar kıyaslanmıştır.

Bu çalışma göstermiştir ki, aseton ile özütlenmiş mantarın antioksidan enzimleri üzerindeki etkisi açıktır ve bu etki kanser ilaçlarıyla birlikte artmaktadır. Metanol ile özütlenmiş mantar ise açık bir etki göstermemiştir. Asetone ile ekstrakt edilmiş Reishi GST enzimin %45 inhibe ederken Doxorubicin ile bu değer %30 değerine ulaşmıştır. Aynı şekilde aseton ile özütlenmiş Reishi Kat enzimini %45 inhibe ederken Doxorubicin varlığında %35 inhibe ettiği bulunmuştur. Metanol ile özütlenmiş mantarlarda hemen hiç bir anlamlı sonuç elde edilememiştir.

## ACKNOWLEDGMENTS

I would like to express my gratitude to all who gave me the possibility to complete this thesis. First and for most I am very thankful to almighty god who gave me health, patience and the strength to be able to do this work.

I would like to thank to my advisor Dr. S. Belgin İşgör who has supported me through my thesis with her patience and knowledge, thank you Dr. İşgör for encouraging my research and her valuable advices.

My appreciation goes to Assoc. Prof. Dr. Yasemin G. İşgör for her invaluable guidance and support during experiments and I would also thank to Dr Yasemin G.İşgör for her friendship and kindly teaching especially for data analysis throughout this study.

I wish to express my thanks to Prof. Dr. Özlem Yıldırım, Prof.Dr. Atilla Cihaner, Assoc. Prof. Dr. Ilgaz Akata and Assist. Prof. Dr. Murat Kaya for their valuable suggestions and corrections.

I want to thank to my biochemistry research laboratory lab-mates and especially Dr. Naznoosh Shomali for her valuable help and support.

Special thanks to my family, words can not express how grateful I am to my mother , my wife Sara and my sons Abdurrahman and Hossam for their sacrifice and patience throughout all my studies.

## TABLE OF CONTENTS

ABSTRACT .....	iii
ÖZ .....	v
ACKNOWLEDGMENTS .....	vi
TABLE OF CONTENTS.....	vii
LIST OF FIGURES .....	ix
LIST OF TABLES .....	x
Chapter 1	
1. INTRODUCTION .....	1
1.1 Glutathione S-Transferase (GST) Enzyme Family.....	1
1.2 Glutathione Peroxidase (GPX) Enzyme Family.....	4
1.3 Catalase (CAT) Enzymem Family.....	6
1.4 Mushrooms.....	7
1.5 Reishi Mushroom.....	8
1.6 Cancer Drugs .....	10
1.7 Scope of This Work .....	12
2. MATERIALS AND METHODS .....	13
2.1 Materials .....	13
2.2 Methods.....	13
2.2.1 Preparation of the Reishi Mushroom Extracts .....	13
2.2.2 Protein Determination .....	14
2.2.3 Determination of Total Phenolic Contents of Reishi Mushroom .....	15
2.2.4 Determination of Flavonoid Contents of Reishi Mushroom.....	16
2.2.5 Glutathione S-Transferase (GST) Enzyme Assay .....	16
2.2.6 Glutathione Peroxidase (GPX) Enzyme Assay .....	18
2.2.7 Catalase Enzyme Assay .....	19

3 RESULTS AND DISCUSSION.....	20
3.1 Total Phenolic Contents and Total Flavonoids Contents .....	20
3.2 Preparation of Doxorubicin (DOX) Drug Concentration.....	20
3.3 Glutathione S-Transferase (GST) Enzyme Activity .....	21
3.4 Glutathione Peroxidase (GPX) Enzyme Activity .....	26
3.5 Catalase (CAT) Enzyme Activity .....	30
4. Conclusion.....	34
References .....	37

## LIST OF FIGURES

Figure 1.1	The Structure of Glutathione S-transferase .....	3
Figure 1.2	The Structure of Glutathione peroxidase .....	4
Figure 1.3	The Mechanism of Principle of GPX assay .....	5
Figure 1.4	The Structure of Catalase .....	7
Figure 1.5	Reishi Mushroom Parts .....	9
Figure 1.6	Doxorubicin Cancer Drug .....	10
Figure 1.7	Scheme of Doxorubicin Interactions with different proteins in cancer cells. ....	11
Figure 2.1	Multi Mode Plate reader and it is 96 well plate.....	17
Figure 3.1	The Effect of Doxorubicin concentration on GST Enzyme Activity ...	21
Figure 3.2	Effect of Mushroom Extracted in Acetone on GST Activity .....	23
Figure 3.3	Effect of Mushroom Extracted in Acetone and Doxorubicin Drug on GST Activity .....	23
Figure 3.4	Effect of Mushroom Extracted in Methanol on GST Activity .....	25
Figure 3.5	Effect of Mushroom Extracted in Methanol and Doxorubicin Drug on GST Activity .....	25
Figure 3.6	Effect of Mushroom Extracted in Acetone on GPX Activity.....	27
Figure 3.7	Effect of Mushroom Extracted in Acetone and Doxorubicin Drug on GPX Activity.....	27
Figure 3.8	Effect of Mushroom Extracted in Methanol on GPX Activity.....	29
Figure 3.9	Effect of Mushroom Extracted in Methanol and Doxorubicin Drug on GPX Activity.....	29
Figure 3.10	Effect of Mushroom Extracted in Acetone on CAT Activity.....	31
Figure 3.11	Effect of Mushroom Extracted in Acetone and Doxorubicin Drug on CAT Activity.....	31
Figure 3.12	Effect of Mushroom Extracted in Methanol on CAT Activity.....	33
Figure 3.13	Effect of Mushroom Extracted in Methanol and Doxorubicin Drug on CAT Activity.....	33

## LIST OF TABLES

Table 2.1 Mushroom Samples Concentrations .....	14
Table 2.2 The Reagent of Lowry Assay.....	15
Table 2.3 The Reaction Components for a Single Reading of GST Enzyme Assay.	17
Table 2.4 The Reaction Components for a Single Reading of GPX Enzyme Assay	18
Table 2.5 The Reaction Components for a Single Reading of CAT Enzyme Assay.....	19
Table 3.1 Total calculated total Polyphenols and Flavonoids contents for Mushroom extracts prepared in acetone and methanol .....	20
Table3.2 The concentration of Mushroom sample used in GST Assay.....	21

## NOMENCLATURE

CAT	Catalase
GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
GPX	Glutathione Peroxidase
CDNB	1-chloro-2,4-dinitrobenzene
DOX	Doxorubicine
BSA	Bovine Serum Albumin
NADPH	Nicotinamide adenine dinucleotide phospho

# **CHAPTER 1**

## **INTRODUCTION**

Drug resistance occurs when drugs have no longer desired clinical effects. Antioxidant enzymes are a group of enzymes responsible for drug resistance especially during cancer. This property of the enzymes leads to use high dose of drugs to show its effect which increases the side effect of drugs. This is assumed to be a natural inbuilt resistance in some individuals and organisms or may arise naturally in the course of treatment. Drug resistance can be linked to a change in either the permeability of the membranes of the organism or enzyme systems of the organism ("Abstracts of original contributions: 43rd Annual Scientific Session," 1994).

Researchers suggested that some mushrooms in combination with commercial anti-cancer drugs works in synergy as an effective tool for combating drug resistance. Mushroom effect on antioxidant enzyme activities which have role in cancer development and chemotherapeutic drug resistance by prevention of the fall of the GSH levels by acting on enzymes involved in the GSH redox cycle ("Abstracts of original contributions: 43rd Annual Scientific Session," 1994).

Studies showed that mushrooms increased the effect of cancer drugs by attacking the antioxidant enzymes and drugs connect with GSH so that the complex get into DNA easily and show the effect of drugs (Backos, Franklin, & Reigan, 2012).

### **1.1 Glutathione-S-transferase (GST) Enzyme Family:**

The glutathione S-transferases (GSTs) belong to phase II detoxifying enzymes and have many other important roles in normal cellular metabolism as well as in the detoxification of a wide variety of xenobiotic compounds (Lu, 2013).

The main role of GST is to catalyze the conjunction of glutathione with electrophilic compounds like nitrogen, sulphur or carbon atoms and to eliminate the foreign compounds including anti-cancer drugs (Lu, 2013).

GSTs have a wide range of substrate specificity which includes nitrobenzenes, epoxides, heterocyclic amines, quinones, arene oxides,  $\alpha$ ,  $\beta$ - unsaturated carbonyls etc (Reinemer, Prade, Hof, Neufeind, Huber, Zettl, et al., 1996).

GST enzymes are evolved from thioredoxin enzymes which are antioxidants and found in a lot of organisms.

GSTs have structure and sequence similarity with other stress related proteins and it is suggested that a common stress related ancestor that is formed before thioredoxin is responsible for this relationship (Reinemer, Prade, Hof, Neufeind, Huber, Zettl, et al., 1996).

GSTs are classified into 5 classes related to their structure and substrates as alpha, ( $\alpha$ ), Mu ( $\mu$ ), Pi ( $\pi$ ), Sigma ( $\sigma$ ) and theta ( $\theta$ ). The active site in these enzymes contain two binding sites called GSH-binding site (G site) and xenobiotic-binding site (H site) ( Parde L, Huber R, Bieseler B, 1998).

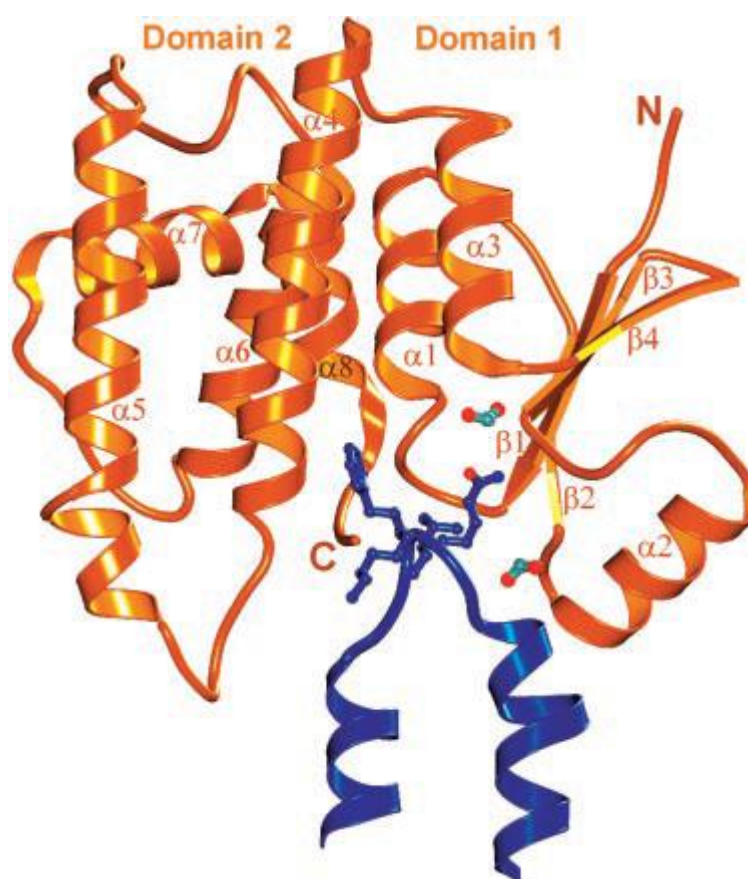
GST consists of 25-30 KDa subunits, which can be divided into two domains, domain N and domain C (Figure 1.1) .

The GST family consists of two superfamilies: the cytosolic and mitochondrial. Cytosolic GSTs are the largest family of transferases and have some special activities; they have thiolysis, reduction and isomerization activities (Hayes *et al.*, 2005).

Besides having these activities, cytosolic GST enzymes can bind covalently and noncovalently to nonsubstrate ligands and have roles in intracellular transport and disposition of xenobiotics. These nonsubstrate hydrophobic ligands are some

steroids, bilirubin, heme and lipophilic anticancer drugs (Hayes and Pulford, 1995).

Mitochondrial GST activity might be present to protect against genotoxic and cytotoxic electrophiles, which is produced within the mitochondria by the activity of mitochondrial cytochrome P450 species or might be result from the decomposition of lipid peroxides produced during respiration (Harris J, Meyer D, Coles B, Ketterer B, 1991).



**Figure 1.1:** The structure of glutathione S-transferase(Wolf K, Backer A *et al.*, 2003).

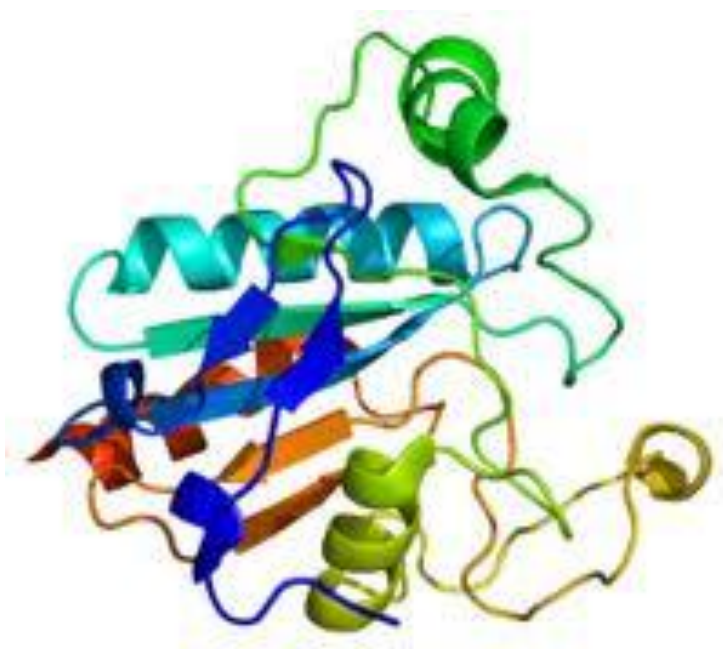
There are evidences that the activity of GST leads to drug resistance, in addition to the elimination of foreign compounds, it has a role in removal of free radicals which are resulted from natural evolution (Board P, Menon D, 2012).

## 1.2 Glutathione Peroxidase (GPX) Enzyme Family:

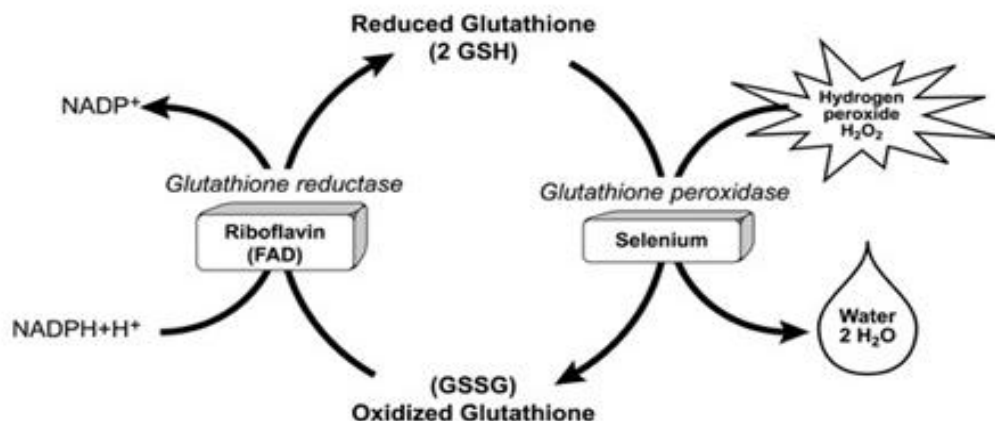
Glutathione peroxidase (GPX) is the general name of enzyme family with peroxide activity, GPX is one of the homotetrameric water soluble antioxidant enzymes (Figure 1.2).

The main biological role of GPX is to protect the organism from oxidative damage, it is responsible for catalysis of reduction of organic hydroperoxides and hydrogen peroxide to alcohols by using Glutathione as a reducing agent (Figure 1.3).

It is one of the seleno protein family observed in mammals, birds and fish(Hayes and McLellan, 1999).



**Figure 1.2:** The structure of GPX (Jurkovic S, Osredkar J, Marc J, 2008)



**Figure 1.3:** The mechanism of principle of GPX assay

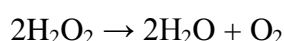
There are at least 7 classes of GPX which have been characterized until now; cytosolic GPX1, gastrointestinal GPX2, plasma GPX3, and phospholipid hydroperoxide GPX4, and GPX6 located in the olfactory epithelium and embryonic tissues, mammalian GPX1, GPX2, GPX3, and GPX4 are selenium-containing enzymes, while GPX6 is a selenoprotein in human with cysteine-containing homologues in rodents (Flohe R and Maorino M, 2013).

Selenium part of GPX active site is in the form of a selenocysteine residue, which is incorporated into the polypeptide backbone (Folh, Rotruck et al., 1973). Mammalian GPX1, GPX2, GPX3 is homotetrameric proteins, whereas GPX4 has monomeric structure as the integrity of the cellular and subcellular membrane depends on GPX (Stadtman, 1991).

### 1.3 Catalase (CAT) Enzyem Family:

Catalase (CAT) has been described from a long time as one of the antioxidant enzymes, it is a protective enzyme present in nearly all animal cells and a lot of plants like barley, cotton, maize, sunflower and tobacco. Its main function is to protect the cells from an oxidative damage of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by converting it to water and oxygen (Afiyanti M, Chen H, 2013).

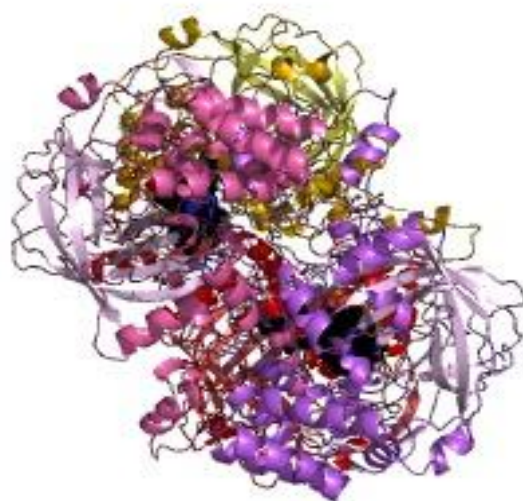
Catalyzed by CAT enzyme is as follows:



CAT is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 KDa that contains a single ferriprotoporphyrin group per subunit, and has a molecular mass about 240 KDa (Figure 1.4) (El-missiry M, 2012).

The catalases are grouped into two parts; small subunit (55 to 69 kda) enzymes containing heme b, and large subunit (75 to 84 KDa) enzymes in which the heme group have flipped through 180° and modified to heme d (Dunford H, 2010).

There are three types of CAT structure: Primary structure, the beef liver catalase monomer consists of polypeptide chain with 506 amino acid plus one heme group and also contains one tightly bound reduced Nicotinamide Adenine Dinucleotide (NADH). Only about 60% of catalase structure is composed of regular secondary and tertiary structures. Each monomer has four domains; the first domain is made up of the amino-terminal 75 residus. These form an arm with two alpha-helices and large loop extending from the globular subunits.



**Figure 1.4:** The structure of CAT ( Bravo J, Verdaguer N, et al., 1995)

#### **1.4 Mushrooms:**

Fungi are a rich and diverse group of organisms on the planet. They have essential roles in ecosystems. Fungi, together with bacteria, are responsible for most of the biodegradation and they are vitally important for the growth of most plants, through the development of mycorrhizal associations. Approximately 100,000 fungi species have been described but it is estimated that 1.5 million species may exist. Macrofungi are one of those fungi that form large fructifications visible without the aid of a microscope and they are defined here to include Ascomycota and Basidiomycota divisions with large, easily observed sporebearing structures.

Most terrestrial macrofungi are saprobes or mycorrhizal symbionts, but some are pathogens of plants or fungi (Mueller et al., 2007; Servi et al., 2010).

Macrofungi is an attractive material to attract the attention of researchers because of its benefits for humans such as strengthening the immune system and anti-cancer. In addition, it contains many nutritional compounds (Zaidman B, et al., 2005).

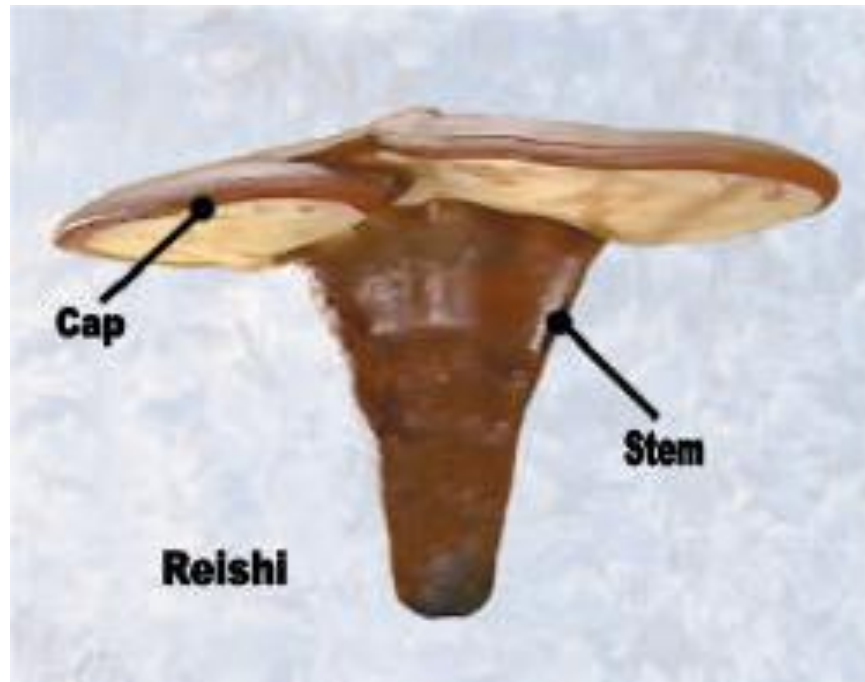
The extraction products of mushrooms are used as food supplements not as a regular food. Food supplements are generally the components extracted from food, plants or fungi which are taken without modification. Growing mushroom is common in China, India and some of developed countries because of a low price of raw material and easily available (Chang S, Miles P, 1989).

Medicinal mushrooms have been used as a traditional treatment in Japan, China and Korea, from the eighties of the last century. Recently many research groups especially in Japan and China have proposed that mushrooms and their extracts increased the drug efficacy as well as lifespan of the patients when introduced as a food supplement during chemotherapy (Zaidman B, et al., 2005).

### **1.5 Reishi Mushroom:**

Reishi (*Ganoderma lucidum*) has been known in China (ling Zhi) more than 2000 years. It was famous as a reinforcement of health superhero and the absence of side effects, which make it distinct from all medicinal herbs. These features made the emperors of China ordering staff to search about it so high in the mountains. Reishi was rare until the late twentieth century, when it was planted by the Japanese who are making it available to the public (Sing W, Moy T, 2003).

Since Reishi is becoming one of the basic food for healthy diet followers, it gathered a lot of information in biological studies of mushroom. Reishi was recommended in China and Japan for insomnia because of enhanced sleep factor, it was given for many mental and neurological pain included muscle diseases, loss of appetite and debility after illness, reishi contains high concentration of organic germanium, sugar and triterpenes, and these ingredients are strengthen the immune



**Figure 1.5:** *Ganoderma lucidum* parts

Reishi has positive effect against asthma, skin inflammation and used in the treatment of stiff neck, stiff shoulders, bronchitis and rheumatism ( Babu P, Subhsreecells. R, 2006).

The studies showed that Reishi is used for chronic hepatitis; it is effective in treatment of liver failure patients.

The studies also showed that anti-cancer ingredient in reishi is Beta D-glucan, which is a polysaccharide and stimulate, or modifies immune system by activating the immune cells as (macrophage) and helper T-cells as well as increasing the immunoglobulin levels to produce high response to foreign cells (bacteria, viruses or tumor cells) ( Babu P, Subhsree R, 2006).

Currently available informations from researches and evidences from animal studies suggest that Reishi have positive impact on cancer patients, especially patients with liver cancer, however, the available information is still very low (Ulbricht C *et al.*, 2010).

### 1.6 Cancer Drugs:

Cancer is a very serious health problem in all developed countries since the beginning of the 21st century and became the main reason for the most common cause of death. Treatment options are surgical removal of the cancer cells, radiation therapy and chemotherapy.

There is a group of important drugs used in the treatment of cancer including Doxorubicin.

Doxorubicin (DOX) (Figure1.7), Daunomycin or Adriamycin is an anthracycline antibiotic, which was isolated firstly from the pigment produced by *Streptomyces peucetius* early in 1960s (Hynek D, Krejcova L *et al.*, 2012).

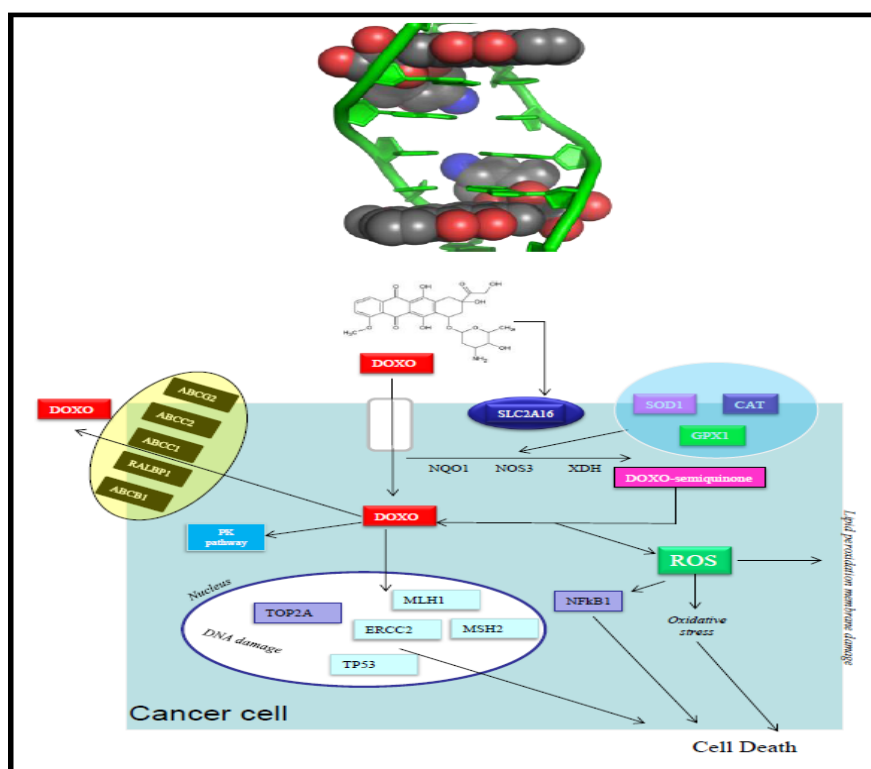


**Figure 1.6:** Doxorubicin cancer drug

DOX is one of the chemotherapeutic drugs given to treat many different types of cancer such as some leukemia, Hodgkin's and non-Hodgkin's lymphomas as well as cancers of the bladder, breast, stomach, lung, ovaries, thyroid, soft tissues and others (Hynek D, Krejcova L et al., 2012).

At present, DOX is commonly used in the treatment of a wide range of cancer but the exact mechanism of the drug is complex and still unclear although it is believed to interact with DNA ( Hynek D, Krejcova L, et al., 2012)

Some experiments were conducted to clarify the differential intracellular toxic effects of DOX on tumor cells and normal cells, these results suggest that DOX has an inhibition effects on tumor cells through the inhibition of DNA synthesis, the toxic effects on tumor cells through the accumulation of reactive oxygen species resulting from low scavenger enzyme activities (Figure 1.7).



**Figure 1.7:** Scheme of DOX interactions with different proteins in a cancer cell (Hynek D, Krejcova L et al., 2012).

### **1.7 Scope of This work:**

Antioxidant enzymes have resistant effects especially on cancer drugs. This leads to the use of excessive doses of medications which may cause side effects; therefore, the aim of this study is to show the effect of Reishi mushroom on antioxidant enzymes such as GPX, CAT and also GST in combination with cancer drug, Doxorubicin.

In this study, the commercially available mushroom pills were used as a mushroom source (Reishi extract-Talya Company, Antalya). The acetone and methanol extractions of mushroom pills were prepared and used in this study.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

#### **2.1 Materials:**

Reishi mushroom pills (Talya), CDNB, 1-chloro-2,4-dinitrobenzene (Fluka analytical), monopotassium phosphate  $\text{KH}_2\text{PO}_4$  (Sigma), Dipotassium phosphate  $\text{K}_2\text{HPO}_4$  (Sigma), hydrogen chloride  $\text{HCl}$  (Fluka), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Acros), dichlorohydroxy benzene sulfonic acid DHBS (Bio Victra), 4-amino antipyrine AP (Sigma), Horse Reddish Peroxidase HRP (Fluka) were purchased from their companies. Bovine liver cytosol were prepared in our laboratory from the bovine liver obtained from slaughter house in Kazan-Ankara.

All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

#### **2.2 Methods:**

##### **2.2.1 Preparation of the Reishi Mushroom Extracts:**

To prepare Reishi mushroom extract, dry mushroom (250 mg) was dissolved in 25 ml of acetone and 25 ml of methanol separately and then the samples are mixed for 30 minutes and kept in the refrigerator for 24 hours. After that the samples were put in a centrifuge for 20 min with the speed of 6000 rpm at  $4^\circ\text{C}$ . After centrifugation, the liquid part is moved to 50 ml evaporation flasks and the solvent is evaporated by using rotary evaporator at  $45^\circ\text{C}$ , finally dry mushroom is dissolved in DMSO (Dimethyl sulfoxide) then stirred carefully and the mushroom extracts are into aliquotes of 1.5 ml and stored in  $-20^\circ\text{C}$  for further analysis. The stock drug plate was prepared by serially diluting the stock samples with DMSO in 2:1.

The mushroom samples and their concentrations (mg/ml) are listed in Table 2.1

**Table 2.1:** The concentrations of mushroom extracts used in the assay protocols.

<b>Dilutions</b>	<b>Mushroom extracted in acetone(mg/ml)</b>	<b>Mushroom extracted in methanol (mg/ml)</b>
1	20	62.5
2	10	31.25
3	5	15.625
4	2.5	7.813
5	1.25	3.906
6	0.625	1.953
7	0.3125	0.977
control	-	-

### **2.2.2 Protein Determination:**

The protein content of the mushroom extracts was determined by the method of Lowry et al., (Lowry 1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1-0.5 ml of 1: 2 diluted sample of mushroom extract were taken into test tubes and were completed to a final volume of 0.5 ml with distilled water. Then alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% potassium sodium tartarate and 0.1 N NaOH containing sodium carbonate in a ratio of 1:1:100, respectively. After that, 2.5 ml of the alkaline copper reagent was added to each tube mixed by a vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 30 minutes at room temperature. The absorbance value was measured at 660 nm.

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding absorbance values of BSA standards (0 to 200 µg) at 660 nm.

The estimation of protein contents in the bovine liver was carried out in duplicate and the results were averaged.

**Table 2.2:** The reagents for Lowry assay.

Content	Concentration
(A).Na <sub>2</sub> CO <sub>3</sub>	2%
(B) Na-K tartarate	1%
(C) CuSO <sub>4</sub>	0.5%
Reagent 1	50 ml of A+1 ml of B+1 ml of C
Reagent 2	1 part of folin-phenol (2 N): 1 part water
BSA	Standard 1mg/ml

The amount of total protein is calculated from the standard calibration curve of BSA as 19.287 mg/ml.

### 2.2.3 Determination of Total Phenolic Contents of Reishi Mushroom:

Folin-Ciocalteu reagents were used for the determination of total polyphenols in the mushroom extract prepared in methanol and acetone at different concentrations and a standard calibration curve was prepared using different concentrations of gallic acid (GA). The absorbance was measured at 750 nm.

Mushroom polyphenols with antioxidant capacity could scavenge reactive chemical species as well as minimizing oxidative damage.

The total phenolic contents of the samples were determined using Folin reagent. On 100 µl mushroom samples, 1 ml of diluted Folin reagent (1:1) was added. After 5 minute incubation in dark 1 ml Na<sub>2</sub>CO<sub>3</sub> was added to the mixture and incubated for 1 h again in the dark at room temperature (The assay samples containing 100 µl DMSO without mushroom extract was used as blank).

The absorbance at 750 nm was measured and total phenolic content was calculated from GA calibration curve prepared as 50-400 mg/ ml.

#### **2.2.4 Determination of Flavonoid Contents of Reishi Mushroom:**

The total flavonoid contents of different crude extracts were estimated by aluminum chloride colorimetric method. 5% sodium nitrate, 4% sodium hydroxide and 10% aluminum chloride( $\text{AlCl}_3$ ) solutions were prepared. 1.25 mL of mushroom crude extracts with different concentrations and sodium nitrate (0.75  $\mu\text{L}$ ) were mixed together. All the test tubes were kept in the dark for 30 min. Then 10% aluminum chloride (0.150  $\mu\text{L}$ ) was added into each test tube incubated for 5 min in the dark to complete the reaction. Finally, 5% sodium hydroxide (0.5 mL) and water (0.275 mL) were added to each test tube. The absorbance of all samples was measured at a fixed wavelength 415 nm. Quercetin standard calibration curve was used for total flavanod contents of mushroom extracts. The estimation of total flavonoids contents in the crude extracts was carried out in triplicate and the results were averaged.

#### **2.2.5 Glutathione-S-Transferase (GST) Enzyme Assay:**

The bovine liver cytosol is used as GST enzyme source and the activity is measured by conjugation of the thiol group of glutathione to the CDNB (1-chloro-2,4-dinitro benzene) substrate by using the miniaturized method of Habig (Habig and Jacoby 1981). The assay conditions are given in Table 2.3.

GST activity was measured by using multi-mode plate reader (Spectra maxM2) at 340 nm (Figure 2.1).

All enzyme assays were carried out in duplicate and conducted at room temperature.



**Figure 2.1:** Multi mode plate reader (Molecular Devices Spectramax M2) and its 96 well plate.

**Table 2.3:** The reaction components for a single reading of GST enzyme assay

Stock Concentrations	Added volume	Final Concentrations
50 mM CDNB	300 $\mu$ l	3 mM
200 mM GSH	100 $\mu$ l	4 mM
200 mM Phosphate buffer (pH 7.0)	4.6 ml	460 mM
Add 200 $\mu$ l mixture from above to plate reader		
Mushroom extract	5 $\mu$ l	
200 mM Phosphate buffer(pH 7.0)	40 $\mu$ l	32 mM
Cytosol (bovine liver cytosol)	5 $\mu$ l	

### 2.2.6 Glutathione Peroxidase (GPX) Enzyme Assay:

GPX activity measurement were based on the measurement of degree of NADPH oxidation at 340 nm with glutathione reductase which uses oxidized glutathione and NADPH as substrate. Since oxidized glutathione is produced by GPX, the degree of NADPH reduction is directly proportional to GPX enzyme activity. The GPX enzyme assay conditions used were given in Table 2.4.

Mixture (glutathione and NADPH) were used as cofactor, submixture (Cumene hydroperoxide, Tris-HCl) provides a suitable reaction and substrate.

**Table 2.4:** The reaction conditions for GPX enzyme assay.

Stock solution	Added amount
<b>Mixture</b>	
20 mM GSH	20 $\mu$ l
20 u/ml GR glutathione reductase	5 $\mu$ l
10 mM NADPH	5 $\mu$ l
<b>Sub mixture</b>	
50 mM Tris-HCl	118 $\mu$ l
30 mM CumOOH (Cumene hydroperoxide)	2 $\mu$ l
50 mM Tris-HCl	PH 8
Added amount	
30 $\mu$ l Mixture , 120 $\mu$ l sub mixture	
Mushroom extracts	4 $\mu$ l
50mM Tris-HCL, pH 8.0	44 $\mu$ l
cytosol (bovine liver cytosolic)	2 $\mu$ l

\*Incubated for 1 minute and read at 340 nm.

### 2.2.7 Catalase (CAT) Enzyme Assay:

In the UV region,  $\text{H}_2\text{O}_2$  shows an increase in absorbance with a shift in wavelength. The dissolution of  $\text{H}_2\text{O}_2$  can be followed by a decrease in absorbance at 240 nm, and the variation in absorbance per unit time is the measure of catalase activity. The assay conditions for determining CAT enzyme activity were given in Table 2.5.

**Table 2.5:** The reaction components for single reading of CAT enzyme assay.

Stock solution	Added amount
Chromogen (in total amount of 5 ml )	
4 AP (4 amino antripyrene)	1 ml
DHBS (dichloro hydroxy benzene sulfonic acid)	1 ml
Kp buffer (Phosphate buffer)	3 ml
For each 1 ml chromogen 5 $\mu\text{l}$ HRB was added	
Added amount in 250 $\mu\text{l}$ drug plate	
Mushroom extract	2 $\mu\text{l}$
100.1 u/ml Catalase	20 $\mu\text{l}$
Phosphate buffer (50 mM)	28 $\mu\text{l}$
10 mM Hydrogen peroxide ( $\text{H}_2\text{O}_2$ )	50 $\mu\text{l}$
Incubation for 2 minutes	
Sodium azide $\text{NaN}_3$ (15 mM)	50 $\mu\text{l}$
Incubation for 5 minutes	
(above mix) + chromogen	5 $\mu\text{l}$ + 255 $\mu\text{l}$
Incubation for 40 minutes and read at 240nm	

\*The reaction mixture without enzyme was read as a blank.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1 Total Phenolic Contents And Total Flavonoids Contents:

The total polyphenols and total flavonoids are determined in both samples of mushroom extracted in acetone and methanol separately. The total phenolic content of acetone and methanol extracted mushrooms are 9.245 and 8.44  $\mu\text{L}/\text{mg}$  and that of flavonoids are 2.140 and 1.841  $\mu\text{L}/\text{mg}$  respectively. The total phenol and flavonoid contents of acetone and methanol extracted mushrooms are given in Table 3.1.

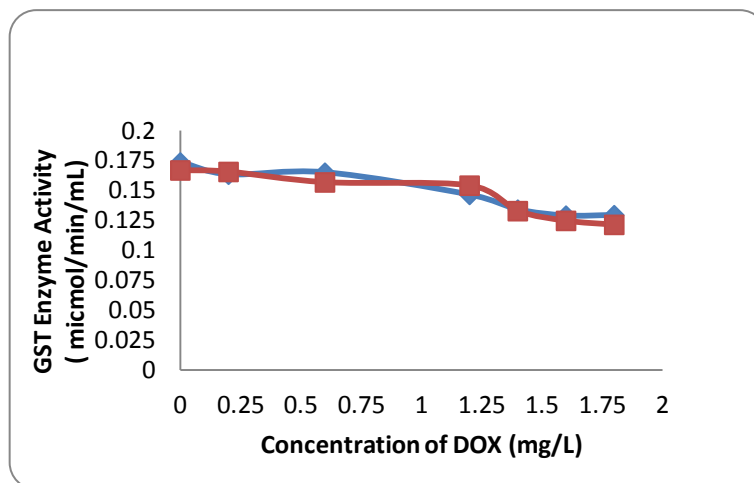
**Table 3.1:** Thecalculated total polyphenol and flavonoid contents for mushroom extracts prepared in acetone and methanol.

Mushroom extracts	Total phenol ( $\mu\text{L}/\text{mg}$ )	Flavonoids ( $\mu\text{L}/\text{mg}$ )
Acetone	9.245	2.140
Methanol	8.44	1.841

#### 3.2 Preparation of Doxorubicin (DOX) Drug Concentration:

Commercially available DOX drug is prepared as it was directed in its prescription as a final concentration of 0.0012 g/L. From the stock solution, four different concentrations of drug as 1200, 400, 133.2 and 44.4  $\mu\text{g}/\text{L}$  were prepared by serial dilution using DMSO as solvent.

The effect of different concentrations of doxorubicin on GST enzyme were tested and the drug concentration of 133.2  $\mu\text{g}/\text{L}$  was used for further analysis(Figure 3.1).



**Figure 3.1** The effect of DOX concentrations prepared (mg/L) on GST enzyme activity.

### 3.3 Glutathione-S-Transferase Enzyme Activity:

Liver cytosol was used as GST enzyme source. GST enzyme activity without mushroom extract was taken as standard and it has a value of 1.1986  $\mu\text{mol/min/mL}$ . Seven different mushroom extracts were prepared for both acetone and methanol extracted samples and tested separately. The concentrations of mushroom extract used in this assay were given in Table 3.2

**Table 3.2:** The concentration of Mushroom samples used in GST assay.

Dilutions	Mushroom extracted in acetone (stock concentrations) (mg/ml)	Mushroom extracted in acetone (assay concentrations) ( mg/ml)	Mushroom extracted in methanol (stock concentrations) (mg/ml)	Mushroom extracted in methanol (assay concentrations) (mg/ml)
1	20	0.4	62.5	1.25
2	10	0.2	31.25	0.625
3	5	0.1	15.625	0.3125
4	2.5	0.05	7.813	0.1562
5	1.25	0.025	3.906	0.0781
6	0.625	0.0125	1.953	0.039
7	0.3125	0.0065	0.977	0.0195

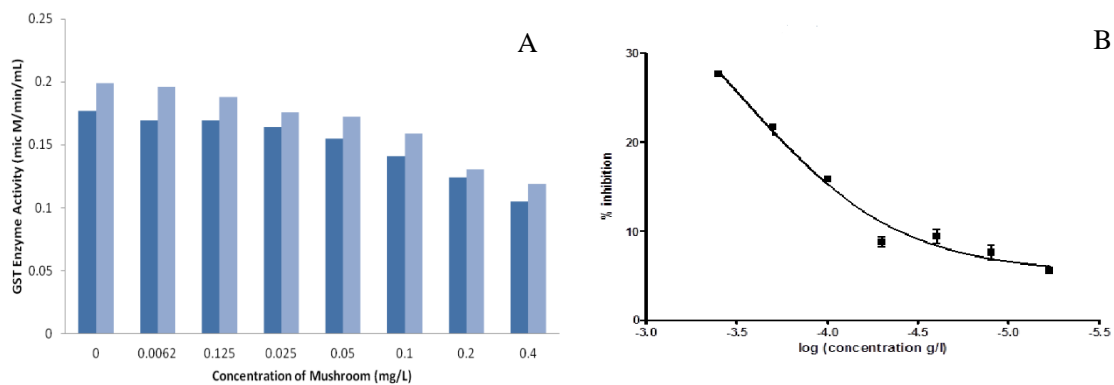
\*Reaction without mushroom extract is taken as blank.

The effect of mushroom extract prepared in acetone on GST enzyme activity towards its substrate CDNB is given in Figure 3.2. The result showed that the GST enzyme activity is inhibited by using 7 different concentrations of mushroom.

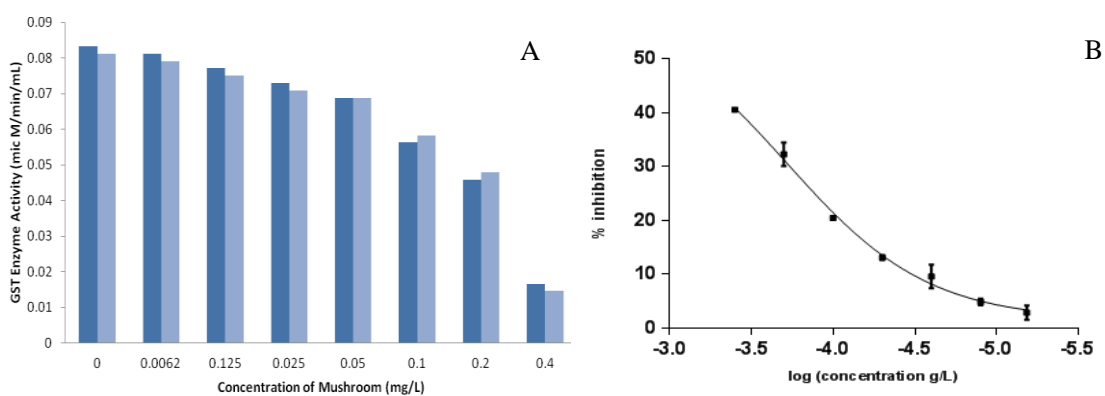
In this graph it was calculated that the GST enzyme activity was decreased about 30% with an increase in concentration of mushroom extract.

The effect of 133.2  $\mu\text{g/L}$  concentration of DOX combined with mushroom, extracted in acetone, on GST enzyme activity towards its substrate CDNB is presented in Figure 3.3. The results showed that the GST enzyme activity is inhibited by using 7 different concentrations of mushroom.

In this graph it was calculated that the GST enzyme activity decreased about 45% with an increase in concentration of mushroom extract.



**Figure 3.2** A) The bar graph presenting the effect of mushroom extracted in acetone on GST enzyme activity. B) Dose-response curve of this effect.

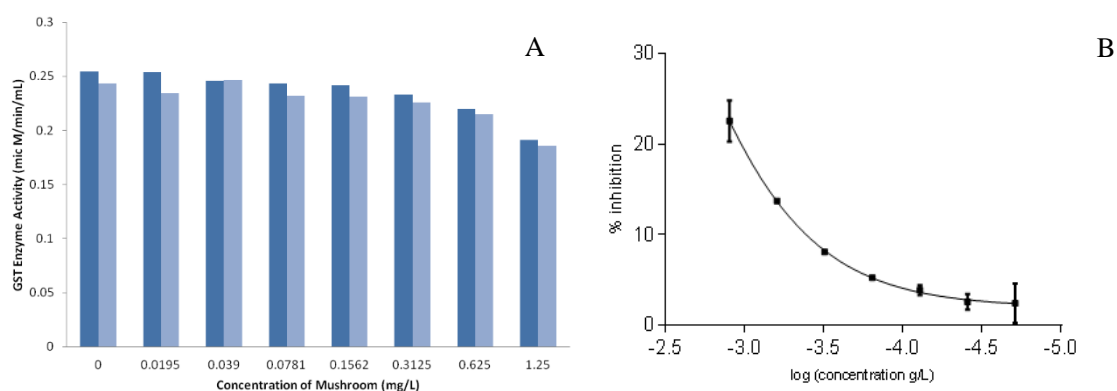


**Figure 3.3** A) The bar graph presenting the effect of mushroom extracted in acetone and DOX drug on GST enzyme activity. B) Dose-response curve of this effect.

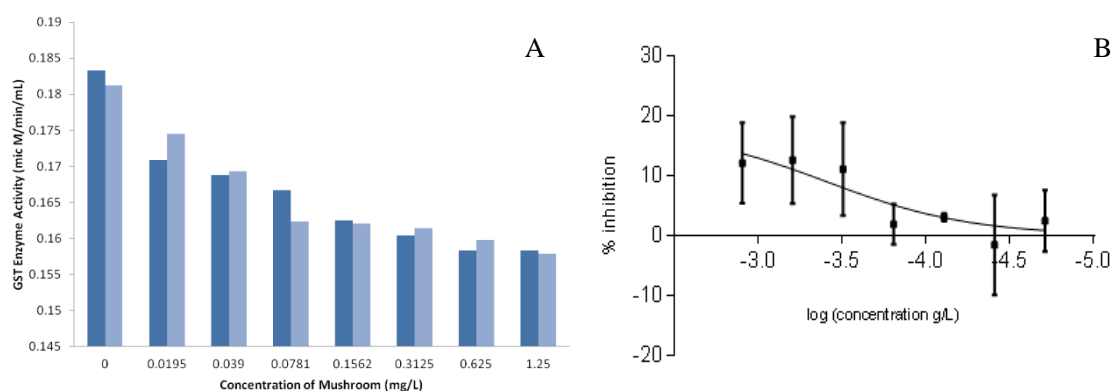
The effect of mushroom extracted in methanol on GST activity towards its substrate CDNB is presented in Figure 3.4. The results showed that the GST enzyme activity decreased about 20% with an increase in concentration of mushroom extract.

The effect of 133.2  $\mu\text{g/L}$  concentration of DOX in combination with mushroom extracted in methanol on GST activity towards its substrate CDNB is given in Figure 3.5.

The results showed that by using 7 different concentrations of mushroom the GST enzyme activity is inhibited 10% with an increase in concentration of mushroom extract.



**Figure 3.4**A)The bar graph presenting the effect of mushroom extracted in methanol on GST enzyme activity.B) Dose-response curve of this effect.



**Figure 3.5**A) The bar graph presenting the effect of mushroom extracted in methanol and DOX drug on GST enzyme activity. B) Dose-response curve of this effect.

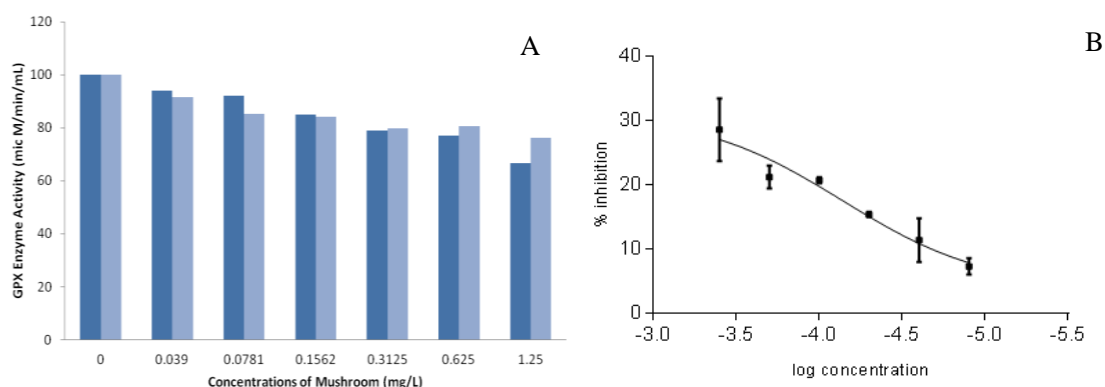
### **3.4 Glutathione Peroxidase (GPX) Enzyme Activity:**

The commercial available enzyme was used as an enzyme source and the cumene hydroperoxide (CumOOH) as substrate. The reduced glutathione, GSH and NADPH were used as cofactor. The GPX activity without mushroom extract and the DOX drug is measured as control and has a value of 1.0142  $\mu\text{mol}/\text{min}/\text{mL}$ . Seven different concentrations of mushroom extracts which are the same as the concentrations used for GST (Table 3.2) were also used here.

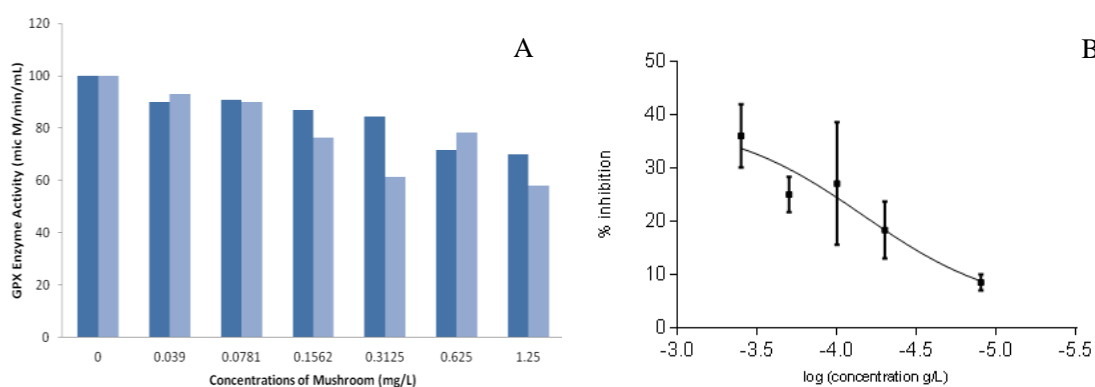
The effect of mushroom extracted in acetone on GPX activity towards its substrate cumenehydroperoxide is presented in Figure 3.6. The results showed that by using 7 different concentrations of mushroom extract the GPX enzyme activity is inhibited about 35% with an increase in concentration of mushroom extract.

The effect of 133.2  $\mu\text{g}/\text{L}$  concentration of DOX in combined with mushroom extracted in acetone on GPX activity towards its substrate cumene hydroperoxide is presented in Figure 3.7. The results showed that the GPX enzyme activity is inhibited by using 7 different concentrations of mushroom.

In this graph it was calculated that the GPX enzyme activity decreased about 15% with an increase in concentration of mushroom extract including with 133.2  $\mu\text{g}/\text{L}$  of DOX.



**Figure 3.6**A)The bar graph presenting theeffect of mushroom extracted in acetone on GPX enzyme activity.B) Dose-response curve of this effect.



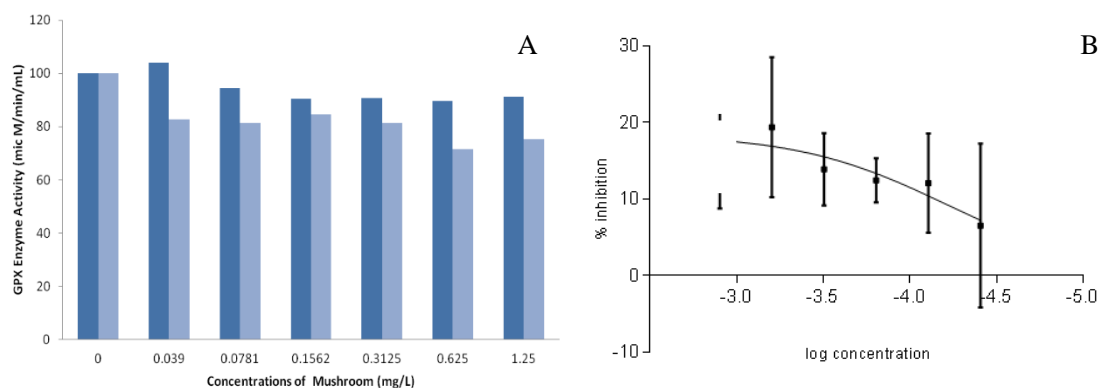
**Figure 3.7**A)The bar graph presenting the effect of mushroom extracted in acetone and DOX drug on GPX enzyme activity. B) Dose-response curve of this effect.

The effect of mushroom extracted in methanol on GPX activity towards its substrate cumene hydroperoxide is presented in Figure 3.8. The results showed that the GPX enzyme activity is inhibited by using 7 different concentrations of mushroom.

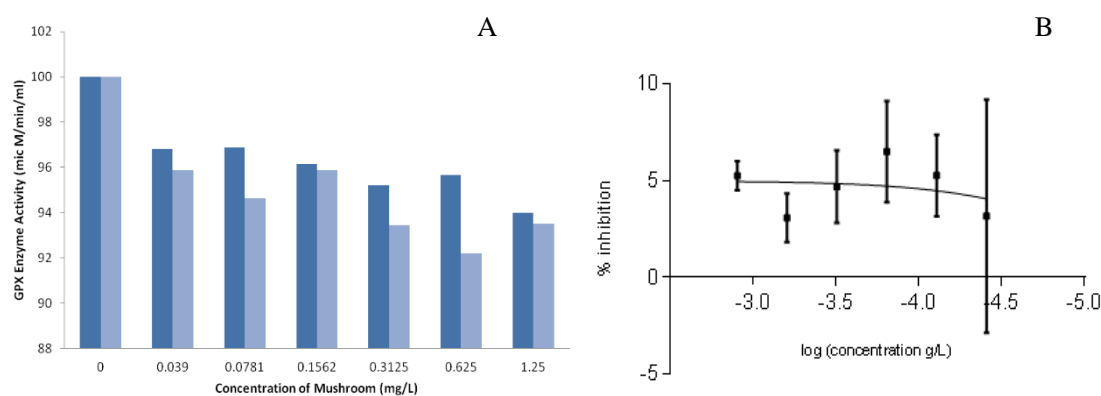
In this graph it was calculated that the GPX enzyme activity decreased about 15% with an increase in concentration of mushroom extract.

The effect of 133.2  $\mu\text{g/L}$  concentration of DOX in combined with mushroom extracted in methanol on GPX activity towards its substrate cumene hydroperoxide is presented in Figure 3.9. The result showed that the GST enzyme activity is inhibited by using 7 different concentrations of mushroom.

Almost no change in GPX enzyme activity has been detected with an increase in concentration of mushroom extract including with 133.2  $\mu\text{g/L}$  of DOX .



**Figure 3.8**A)The bar graph presenting the effect of mushroom extracted in methanol on GPX enzyme activity. B) Dose-response curve of this effect.



**Figure 3.9**A)The bar graph presenting the effect of mushroom extracted in methanol and DOX drug on GPX enzyme activity.B) Dose-response curve of this effect.

### 3.5 Catalase (CAT) Enzyem Activity:

The commercial available enzyme was used as a CAT enzyme source. Hydrogenperoxide was used as a substrate. The CAT activity without mushroom extract and the DOX drug is measured as control and has a value of 1.3263  $\mu\text{mol}/\text{min}/\text{ml}$ .

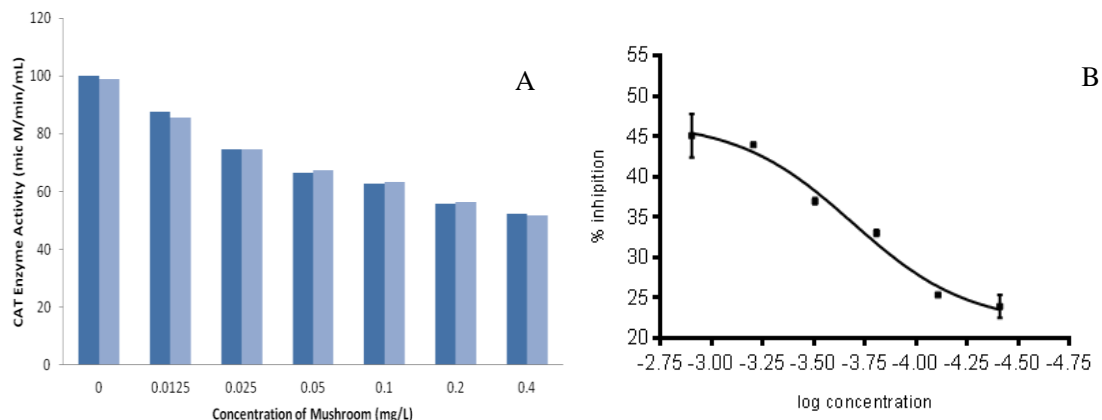
The CAT activity without mushroom extract and the DOX drug is measured as a control and has a value of 1.0142  $\mu\text{mol}/\text{min}/\text{ml}$ . Seven different concentrations of mushroom extracts are the same as the concentrations used before (Table 3.2) were also used here.

The effect of mushroom extracted in acetone on CAT activity towards its substrate hydrogenperoxide is presented in Figure 3.10. The result showed that the CAT enzyme activity is inhibited by using 7 different concentrations of mushroom.

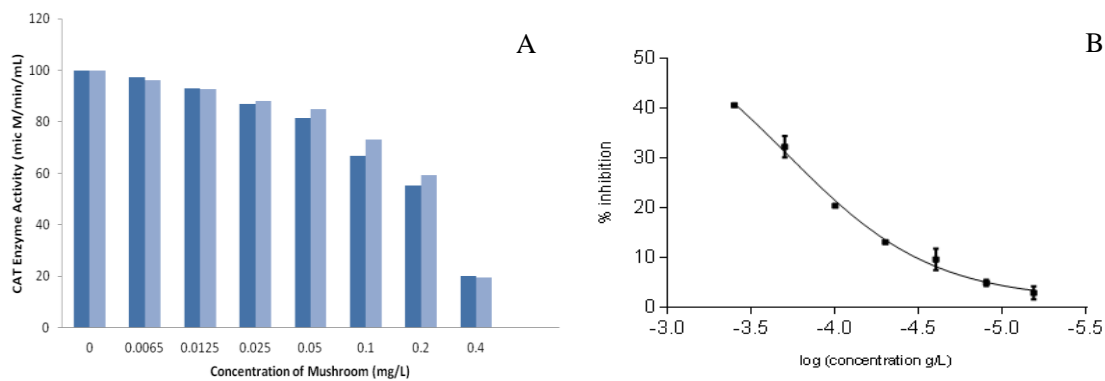
In this graph it is calculated that the GPX enzyme activity decreased about 35% with an increase in concentration of mushroom extract.

The effect of 0133.2  $\mu\text{g}/\text{L}$  concentration of DOX in combined with mushroom extracted in acetone on CAT activity towards its substrate hydrogenperoxide is presented in Figure 3.11. The results showed that the GST enzyme activity is inhibited by using 7 different concentrations of mushroom.

In this graph it was calculated that the CAT enzyme activity decreased about 45% with an increase in concentration of mushroom extract including with 133.2  $\mu\text{g}/\text{L}$  of DOX.



**Figure 3.10** A)The bar graph presenting the effect of mushroom extracted in acetone on CAT enzyme activityB) Dose-response curve of this effect.



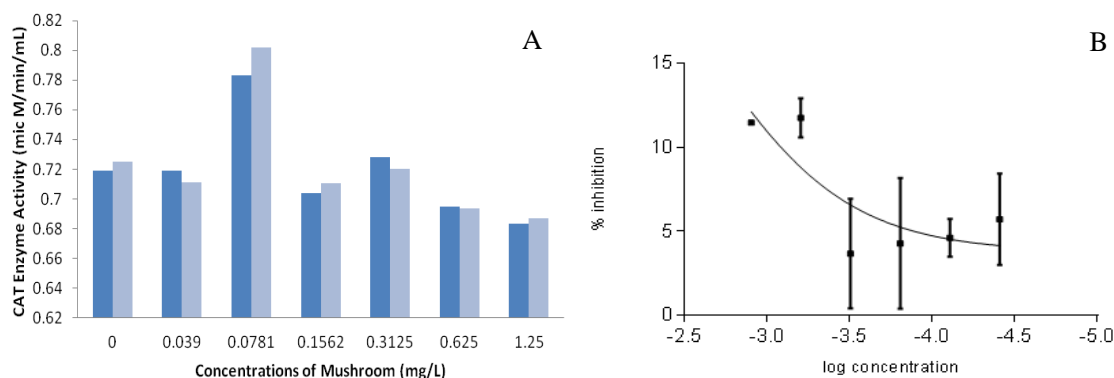
**Figure 3.11**A)The bar graph presenting the effect of mushroom extracted in acetone and DOX drug on CAT enzyme activity.B) Dose-response curve of this effect.

The effect of mushroom extracted in methanol on catalase activity towards its substrate hydrogenperoxide is presented in Figure 3.12. The result showed that the CAT enzyme activity is inhibited by using 7 different concentrations of mushroom.

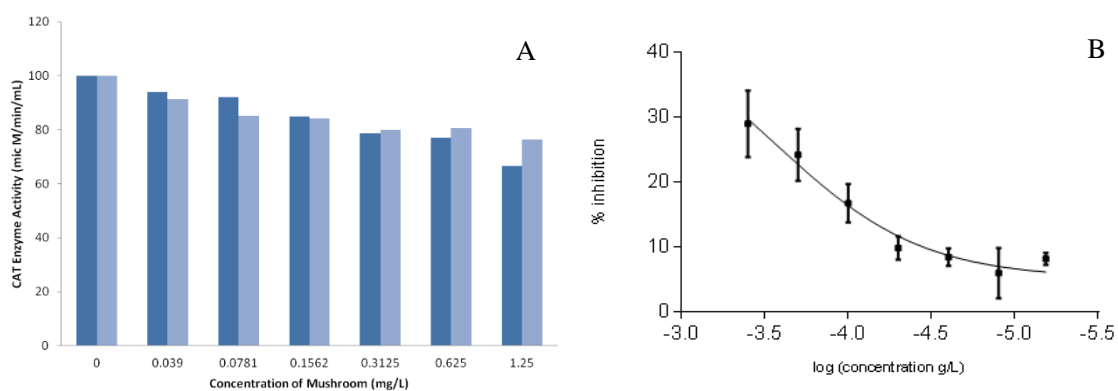
In this graph no significant CAT enzyme activity result was obtained with an increase in the concentration of mushroom extract.

The effect of 133.2  $\mu\text{g/L}$  concentration of DOX in combined with mushroom extracted in methanol on CAT activity towards its substrate hydrogenperoxide is presented in Figure 3.13. The results showed that the CAT enzyme activity is inhibited by using 7 different concentrations of mushroom.

In this graph it was calculated that the CAT enzyme activity decreased about 20 % with an increase in concentration of mushroom extract including with 133.2  $\mu\text{g/L}$  of DOX.



**Figure 3.12**A)The bar graph presenting the effect of mushroom extracted in methanol on CAT enzyme activity.B) Dose-response curve of this effect.



**Figure 3.13**A)The bar graph presenting the effect of mushroom extracted in methanol and DOX drug on CAT enzyme activity. B) Dose-response curve of this effect.

## CONCLUSION

In this study commercially available Reishi mushroom extract was used to test its effect on four different antioxidant enzymes namely GST, GPX and CAT with or without Doxorubicin drug.

Superoxide dismutase (SOD) enzyme was tested also in this study but it is eliminated because it did not gave results.

During this study, Reishi mushroom extracts were prepared by using five different solvents (H<sub>2</sub>O, DMSO, methanol, ethanol and acetone). After preparing the extract and evaporating the solvent the remained sample were dissolved in DMSO for further analysis. It has observed that the solvent was turbid with H<sub>2</sub>O, DMSO and ethanol extracted mushroom. Due to the solubility problem with H<sub>2</sub>O, DMSO and ethanol these solvents were excluded from the study. It has decided to use methanol and acetone for further studies.

DOX cancer drug were studied by comparing their effects on antioxidant enzymes Glutathione S Transferase, Glutathione peroxidase and Catalase.

It has been shown that the GST activity was inhibited with the use of mushroom extract prepared in acetone by 30% while the inhibition with the methanol extracted samples was 20%. also it has concluded that effect of acetone extracted mushroom on GST enzymes was better than the effect of methanol extracted mushroom.

The experimental results in this study showed that the CAT enzyme activity the was inhibited by 35% with the use of acetone extracted mushroom. On the other hand, no significant results were obtained with methanol extracted sample.

In present study it has calculated that GPX enzyme activity was inhibited 35%

and 15% by the use of acetone and methanol extracted mushrooms respectively

.

In this study, the effect of cancer drug DOX on the activity of the enzymes GST, GPX and CAT in combined with the mushroom extracts prepared in acetone was also tested.

After comparing the results obtained from the mushroom extracted in methanol and acetone on the enzyme activities it has concluded that the methanol extracted mushrooms showed no significant inhibition ( $\leq 20\%$ ) with the enzymes studied. The methanol extracted mushroom resulted 20% and 15% inhibition with GST and GPX respectively and did not give any result with the CAT enzyme. So it has decided to use only acetone extracted mushroom combined with DOX. After comparing the results of acetone extracted mushrooms on enzyme activity with and without DOX, it has concluded that much higher inhibition of the enzyme activities were obtained with the mushroom extracts together with cancer drug DOX.

The results showed that the GST enzyme inhibition by mushroom extracted acetone with DOX was 45% while it was 30% when the mushroom extracts were used alone.

With the use of drug DOX, the inhibition of CAT activity was increased to 45% from 35%.

It has shown that the use of acetone extracted mushroom combined with cancer drug DOX did not change the inhibition result of the enzyme obtained from the acetone extracted mushroom alone. Both experiment with or without DOX the acetone extracted mushroom inhibited enzyme activity by 35%.

In this study it has concluded that the extracted Reishi mushroom combined with the cancer drug, the GST enzyme activity responsible for drug resistance was inhibited more. On the other hand, the Reishi mushroom extract combined with DOX did not change the CAT enzyme activity which is responsible for preventing cells from oxidative damage.

Although further tests were required after comparing the experimental results obtained throughout this study, the Reishi mushroom extract can be used as food supplement to increase the drug effect in combination with cancer drug DOX.

## References

- Abstracts of original contributions: 43rd Annual Scientific Session. (1994). Journal of the American College of Cardiology, 23(2, Supplement 1), 3A-484A.
- Afiyanti, M., & Chen, H.-J. (2014). Catalase activity is modulated by calcium and calmodulin in detached mature leaves of sweet potato. *Journal of Plant Physiology*, 171(2), 35-47.
- Backos, D. S., Franklin, C. C., & Reigan, P. (2012). The role of glutathione in brain tumor drug resistance. *Biochemical Pharmacology*, 83(8), 1005-1012.
- Board, P. G., & Menon, D. (2013). Glutathione transferases, regulators of cellular metabolism and physiology. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830(5), 3267-3288.
- Boh, B., Berovic, M., Zhang, J., & Zhi-Bin, L. (2007). Ganoderma lucidum and its pharmaceutically active compounds. In M. R. El-Gewely (Ed.), *Biotechnology Annual Review*, vol. Volume 13 (pp. 265-301): Elsevier.
- Bravo, J., Verdager, N., Tormo, J., Betzel, C., Switala, J., Loewen, P. C., & Fita, I. (1995). Crystal structure of catalase HPII from *Escherichia coli*. *Structure*, 3(5), 491-502.
- Brigelius-Flohé, R., & Maiorino, M. (2013). Glutathione peroxidases. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830(5), 3289-3303.
- Chroust, K., Jowett, T., Farid-Wajidi, M. F., Huang, J.-Y., Ryskova, M., Wolf, R., & Holoubek, I. (2001). Activation or detoxification of mutagenic and carcinogenic compounds in transgenic *Drosophila* expressing human glutathione S-transferase. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 498(1-2), 169-179.
- Eckelt, V. H. O., Liebau, E., Walter, R. D., & Henkle-Dührsen, K. (1998). Primary sequence and activity analyses of a catalase from *Ascaris suum*. *Molecular and Biochemical Parasitology*, 95(2), 203-214.
- Hynek, D., Krejcova, L., Zitka, O., Adam, V., Trnkova, L., Sochor, J., Stiborova, M., Eckschlager, T., Hubalek, J., & Kizek, R. (2012). Electrochemical study of Doxorubicin interaction with sequences of single stranded oligonucleotides, *Journal of Electrochemical science* ;7:13-33.
- Jean Lodge, D., Ammirati, J. F., O'Dell, T. E., Mueller, G. M., Huhndorf, S. M., Wang, C.-J., Stokland, J. N., Paul, J., Leif Ryvarden, S., Leacock, P. R., Mata, M., Umaña, L., Wu, Q. F., & Czederpiltz, D. L. (2004). 8 - Terrestrial and Lignicolous Macrofungi. In G. M. M. F. B. S. Foster (Ed.), *Biodiversity of Fungi*, (pp. 127-172). Burlington: Academic Press.
- Jones, P., & Dunford, H. B. (1977). On the mechanism of compound I formation from peroxidases and catalases. *Journal of Theoretical Biology*, 69(3), 457-470.

- Ketterer, B., & Christodoulides, L. G. (1994). Enzymology of Cytosolic Glutathione S- Transferases. In M. W. Anders & D. Wolfgang (Eds.), *Advances in Pharmacology*, vol. Volume 27 (pp. 37-69): Academic Press.
- Ketterer, B., Meyer, D. J., Lalor, E., Johnson, P., Guengerich, F. P., Distlerath, L. M., Reilly, P. E. B., Kadlubar, F. F., Flammang, T. J., Yamazoe, Y., & Beaune, P. H. (1991). A comparison of levels of glutathione transferase, cytochromes P450 and acetyltransferases in human livers. *Biochemical Pharmacology*, 41(4), 635-638.
- Kurokawa, S., Eriksson, S., Rose, K. L., Wu, S., Motley, A. K., Hill, S., Winfrey, V. P., McDonald, W. H., Capecchi, M. R., Atkins, J. F., Arnér, E. S. J., Hill, K. E., & Burk, R. F. (2014). Sepp1UF forms are N-terminal selenoprotein P truncations that have peroxidase activity when coupled with thioredoxin reductase-1. *Free Radical Biology and Medicine*, 69(0), 67-76.
- Lu, S. C. (2013). Glutathione synthesis. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830(5), 3143-3153.
- Maiorino, M., Ursini, F., Bosello, V., Toppo, S., Tosatto, S. C. E., Mauri, P., Becker, K., Roveri, A., Bulato, C., Benazzi, L., De Palma, A., & Flohé, L. (2007). The Thioredoxin Specificity of Drosophila GPX: A Paradigm for a Peroxiredoxin-like Mechanism of many Glutathione Peroxidases. *Journal of Molecular Biology*, 365(4), 1033-1046.
- Mueller GM, Schmit JP, Leacock PR, Buyck B, Cifuentes J, DesjardinDE, Halling RE, Hjortstam K, Iturriaga T, Larsson KH, Lodge DJ, May TW, Minter D, Rajchenberg M, Redhead SA, Ryvarden L, Trappe JM, Watling R, Wu Q (2007). Globaldiversityanddistribution of macrofungi. *Biodivers. Conserv.* 16: 37-48.
- Oakley, A. J. (2005). Glutathione transferases: new functions. *Current Opinion in Structural Biology*, 15(6), 716-723.
- Prade, L., Huber, R., & Bieseler, B. (1998). Structures of herbicides in complex with their detoxifying enzyme glutathione S-transferase – explanations for the selectivity of the enzyme in plants. *Structure*, 6(11), 1445-1452.
- Reinemer, P., Prade, L., Hof, P., Neufeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Bartunik, H. D., & Bieseler, B. (1996). Three-dimensional structure of glutathione S-transferase from *Arabidopsis thaliana* at 2.2 Å resolution: Structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture. *Journal of Molecular Biology*, 255(2), 289-309.
- Stadtman, E. R. (2002). Importance of individuality in oxidative stress and aging. *Free Radical Biology and Medicine*, 33(5), 597-604.

- Yan, F., Yang, W.-k., Li, X.-y., Lin, T.-t., Lun, Y.-n., Lin, F., Lv, S.-w., Yan, G.-l., Liu, J.-q., Shen, J.-c., Mu, Y., & Luo, G.-m. (2008). A trifunctional enzyme with glutathione S-transferase, glutathione peroxidase and superoxide dismutase activity. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1780(6), 869-872.
- Zaidman, B. Yassin, M. Mahajna, J. Wasser, S. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics, *Biotechnology Annual Review*, ;67:453-468.